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(54) PROCESS FOR THE CRYOGENIC PRESERVATION OF BLOOD AND ERYTHROCYTES AND PRODUCTS PRODUCED THEREBY

(71) We, AMERICAN HOSPITAL SUPPLY CORPORATION, a Corporation organized existing under the laws of the State of Illinois, United States of America, of 1740 Rodge Avenue, Evanstown, Illinois, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

In the cryogenic processes for the preservation of human blood, or erythrocyte-containing fractions thereof, the principal problem is to avoid or at least minimize the destruction of the erythrocytes (red blood cells), which are subject to membrane damage or hemolysis even with the most advanced techniques of freezing and thawing, such as the Linde liquid-nitrogen technique, or the improvement of this process for bulk processing of blood and erythrocytes as disclosed in United States Patent 3,347,745. It has been found essential to employ a cryophylactic agent to protect the erythrocytes during freezing and thawing, and there has been a continuing search for better protective agents. Intracellular protective agents, such as glycerol, have been effectively used with long-term preservation of erythrocytes, but they suffer from a serious disadvantage. It is necessary to remove the intracellular protective agent from the erythrocytes prior to administration.

It has been recognized that an extracellular protective agent providing effective protection and at the same time being administrable with the cells would have a definite advantage in reducing to a minimum the processing after thawing. Certain extracellular protective additives have not been considered safe for administration to humans. For example, polvinylpvrrolidone (PVP) has been found to be highly effective in the preservation of erythrocytes, but there is a recognized hazard in introducing PVP into the vascular system. The PVP, at least the larger-molecular weight fractions thereof, may persist in the body for periods of time, and such PVP retention is considered to be undesirable. In general, to remain outside the cells, the protective agent must be a polymer of relatively long chain length, and therefore the problem of long term retention in the body has been believed to be inherent in extra-cellular protectants.

Ordinary unmodified starch or other saccharide polymers subject to amylolysis are attacked by blood amylases and are therefore not effective protectants. The erythrocytes are usually frozen in the presence of blood amylases. The problem is particularly acute with respect to whole blood, but it also applies to erythrocytes suspended in an aqueous medium which is at least partially blood plasma.

It has been previously proposed that hydrolyzed etherified starch be employed as a protective additive for the cryogenic preservation of human blood and erythrocytes. However, prior to the present invention it was believed that high ether group substitution levels were required. For example, modified starches previously found useful for freeze-preservation of erythrocytes contain from 0.6 to 0.8 (viz. 0.75 hydroxyethyl or hydroxypropyl groups per glucose mole. Furthermore, it was believed that if the ether group substitution was reduced to a substantially lower level, then the effectiveness of the product as a preservative would be substantially reduced. This invention is therefore based on the unexpected discovery that hydrolyzed etherified waxy starches containing low substitution level of ether groups are efficient and effective preservatives for blood and erythrocyte. The invention therefore makes possible an improved process wherein the blood and erythrocytes are protected and preserved during the cryogenic process while permitting the blood and erythrocytes to be administered without removal of the starch protectant, and with a minimization of side effects due

to the persistence of the starch protectant in the blood stream. At the low substitution levels employed in the present invention, the starch is rapidly depleted from the blood stream, and this desirable effect is further enhanced when the starch has been hydrolyzed to a low inherent viscosity.

It has been further discovered that the protective action of the low substituted starches can be maximized (as compared with high substituted starches) by a very small increase in concentration of the starch in the blood or erythrocyte suspension, for example, an increase from 12% to 15%. Thus, the advantage of minimal intravascular persistence can be obtained without sacrifice of protective effectiveness, and, in fact, obtaining the maximal protective action that is possible at any degree of ether group substitution up to as high as 0.75 D.S.

The preferred starting material for practicing the present invention is waxy starch in granule form. For example, waxy milo (sorghum) starch, waxy maize starch, or waxy rice starch can be used. Waxy starches for use in the present invention preferably contain 90% or more by weight of amylopectin. Pregelatinized waxy starches can be used, but it has been found convenient to gelatinize the starch immediately prior to or concurrently with the hydrolysis. In other words, the gelatinization and hydrolysis can be one continuous processing step. Waxy starches which are very fluid when boiling are particularly suitable, such as those having fluidities of 85 or above. It will be understood that the starch starting material will have a substantially higher inherent viscosity than the desired product after hydrolysis.

For the starches not pregelatinized, the acid hydrolysis conditions are effective for complete gelatinization. Following gelatinization the starch is subjected to acid hydrolysis to reduce its inherent viscosity. The gelatinization and hydrolysis is carried out with the starch in a water suspension. The suspension concentration of the starch is not particularly critical, suitable ratios of starch to H_2O ranging from about 0.65 to 0.75 on a weight:weight basis. The suspension is preferably at a low acid pH, such as a pH from 2.0 to 3.0. The pH can be adjusted with various acids, such as hydrochloric acid or sulphuric acid. The rate of hydrolysis will depend on the temperature. A suitable temperature range is from 85 to 95° C. The hydrolysis preferably proceeds sufficiently slowly so that its progress can be followed analytically, permitting the hydrolysis to be terminated at the selected end point. By taking a series of samples as the hydrolysis proceeds, the time required for completion of the hydrolysis can be determined by extrapolation with rather close accuracy.

Various known procedures may be used for determining inherent viscosity. For example, the viscosity in deciliters (dl) per gram (gm) may be determined by flow time in an Ubellohde viscometer, and the measured viscosity corrected for concentration as measured by optical rotation or by the anthrone colorimeter method. The details of suitable analytical procedures will be further described following the specific examples.

The extent of hydrolysis determines the inherent viscosity of the final product. It is therefore advisable to preselect the viscosity end point of the hydrolysis step. The final viscosity should not be over 0.27 dl/gm at 25° C., and preferably not over 0.24—0.25 dl/gm at 25° C. The hydrolyzed product should retain its character as starch, which it does down to inherent viscosities of as low as 0.07—0.10 dl/gm at 25° C. For maximizing the advantages of the present invention the optimum I.V. range is 0.13 to 0.17 dl/gm at 25° C.

The starch either before or after hydrolysis is etherified by reaction with ethylene or propylene oxide, but ethylene oxide is preferred. The etherification should be conducted under basic pH conditions, since the presence of an alkali such as sodium hydroxide, is desirable to promote the etherification reaction. The etherification may be conducted at a pH of from about 11 to 13, with an alkali concentration of from about 8 to 10% based on the starch solids. The temperature of the reaction mixture during the etherification is preferably substantially lower than during the hydrolysis. While the etherification can be carried out at temperatures ranging from 35 to 70° C., temperature conditions of the etherification step should be selected to promote the etherification and achieve the desired substitution level without materially changing the inherent viscosity of the starch. Any hydrolysis of the starch occurring during the etherification should be negligible.

In the etherification step, the same concentration of starch in the suspension can be used as in the hydrolysis step. Consequently, after the conclusion of the hydrolysis, the reaction mixture can be prepared for the etherification by cooling and the addition of sodium hydroxide or other alkali. Preferably, however, the starch is etherified prior to hydrolysis. This sequence has been found to greatly reduce the formation of colored by-products, and therefore no sodium bisulfite or activated carbon treatment are required to produce a white product or colorless solution.

A suitable analytical test for determining

substitution level is set out following the specific examples. In accordance with the present invention, the substitution should be selected so that the D.S. is less than 0.5 and preferably above 0.20. The optimum range is 0.25 to 0.45 D.S. The etherified starch will then contain from 0.25 to 0.45 molecularly combined ether groups (viz. hydroxyethyl or hydroxypropyl) per unit of starch (anhydroglucose unit).

The etherified and hydrolyzed product can be purified to remove glycol and other by-products. For example, ethylene glycol or propylene glycol can be removed by an acetone extraction, or the product can be purified by dialysis. Preferably, the purified material will contain less than 0.5% by weight of glycol after drying. The product can be converted to the form of a dry powder by conventional techniques such as drum drying or spray drying.

In utilizing the extracellular protective agent of this invention, all that is required is to incorporate it in the whole blood or in the aqueous medium in which the erythrocytes are suspended in a sufficient concentration to protect the erythrocytes during freezing and thawing. The amount employed is not critical providing there is a sufficient concentration to perform the protective function. However, since the blood or erythrocytes are to be administered without removal of the starch protectant, it is preferred to employ a minimum amount of the starch while still achieving optimum or substantially complete protection of the erythrocytes. The optimum level will vary depending on whether the cryogenic process is applied to whole blood, or to the separated erythrocytes, which are suspended in an aqueous medium either in the same or a different concentration than in the whole blood. When the erythrocytes are centrifugally separated before being subjected to freezing, they should be resuspended in a suitable aqueous medium, such as a sterile normal saline solution. The aqueous medium containing the starch protectant should be in contact with the membranes of each cell, and this can be most readily obtained by a suitable suspension of the cells in the aqueous medium containing the protectant.

For effective protection of whole human blood, the cryo-protectant of this invention can be employed at a concentration within the range from 13 to 17% w/v. For example, a concentration of 15 grams of the etherified, hydrolyzed starch per 100 milliliters of the additive-containing whole blood can be used. The optimum level with whole blood is about 14 to 16% w/v.

As previously indicated, this invention can be employed with rapid freezing cryogenic processes for erythrocyte protection where the erythrocytes are suspended in blood or an aqueous medium, permitting the protectant to be incorporated in the medium prior to freezing. The so-called "Linde liquid nitrogen technique" for erythrocyte protection is well known in the art and has been thoroughly described in the literature. See P. W. Gikas, C. T. Knorpp, N. W. Thompson, W. R. Merchant, in *Proc. Congr. Int. Soc. Blood Transfus. 10th Stockholm 1964* (Karger, Basel and New York, 1965), pp. 714—718; N. W. Thompson, C. T. Knorpp, P. W. Gikas, M. A. Tinker, W. R. Merchant, ibid., pp 719—725; C. T. Knorpp, P. W. Gikas, N. Thompson, *Cryobiology* 2, 268 (1966).

A suitable procedure for bulk preservation of blood and erythrocytes is described in United States Patent No. 3,347,745, issued October 17, 1967, and entitled "Process for Freezing Erythrocytes". The blood or erythrocytes will be thawed in the same manner as disclosed in the cited references and is well known in the art. The principal difference in the process of this invention following the thawing is that no procedures are required for removal of part or all of the starch protectant.

This invention is further illustrated by the following specific examples.

Example I

1560 gm. of thin-boiling waxy sorghum starch was suspended in 2190 ml. of water and added to the reactor, which was then sealed. The agitator was turned on, and the reactor alternately evacuated and filled twice with nitrogen 385 ml, of 9.2*N* sodium hydroxide was added and the reactor was again evacuated and filled twice with nitrogen. After evacuation to 25 inches Hg. 100 gms. of ethylene oxide was added from a gas cylinder at a rate such that the pressure did not exceed 10 PSI. The reactor was heated by passing 45° C. water through the jacket. When the reactor temperature reached 55° C., it was then maintained 1 hour at 45—50° C. The substituion achieved was 0.30.

572 ml. of 6.2 *N* hydrochloric acid was added, a sample was drawn for pH measurement, and the pH adjusted to 2.0 by further addition of sodium hydroxide or hydrochloric acid as required. Then the reactor was heated with steam until the temperature reached 90° C. Samples were removed at ½ hour intervals for determination of inherent viscosity. Water can be used as the diluent for the determinations. When the estimated inherent viscosity was 0.15 dl/gm, the reaction was terminated by cooling the reactor with cold tap water. After cooling to 30° C., the product was removed and stored under nitrogen at 4° C.

510 ml. of the above syrup was mixed

with 153 ml. of water and 867 ml. of acetone for 15 minutes. After standing for 45 minutes, during which two layers separated, the upper (acetone-rich) layer was siphoned off. The lower (HES-rich) layer was mixed with 246 ml. water and 574 ml. acetone and stirred 15 minutes. After 45 minutes standing, the upper layer was siphoned off and the lower layer was mixed with 246 ml. water and 574 ml. acetone for 15 minutes. After 45 minutes standing, the upper layer was discarded and the lower layer was mixed with 123 ml. water and 697 ml. acetone for 15 minutes. After 45 minutes standing the upper layer was discarded.

400 ml. of water was added to the lower layer, and residual acetone was removed by heating for 15 minutes at 80° C. in a rotary evaporator, using a vacuum of approximately 20 inches Hg. The product was stored at 4° C.

Example II

An HES of IV 0.25 and DS 0.36 is prepared as described in Example I, with the following changes:

A. 180 gms of ethylene oxide is used, instead of the 100 gms of Example I.
B. The hydrolysis reaction is terminated by cooling the reactor when the estimated IV is 0.25, instead of 0.15 as specified in Example I.

Example III

Thin-boiling waxy sorghum starch (238 gm) is stirred with 335 ml. of water and 3.0 ml. of 1.1N HCL in a 1-liter resin flask fitted with a stirrer, dropping funnel, sampling tube, and manifold leading to a water aspirator and a nitrogen tank. The flask is alternately filled with nitrogen and evacuated a total of 3 times. The flask is then heated to 90° C. in a water bath. At 30 minute intervals samples are withdrawn, and the inherent viscosity of the starch determined. When the IV is calculated to be 0.15, by extrapolation, the reaction is terminated by adding 3 ml. of 1N NaOH and cooling to 22°.

Fifty-five ml. of 10.2 N NaOH is added, and then 48 ml. of propylene oxide is added slowly through the dropping funnel. The flask is then heated to 62° and maintained at this temperature for 40 minutes. Then 90 ml. of 6N HCl is added, the syrup is removed and stored under nitrogen at 4° C.

The syrup (700 ml.) is stirred with 1.21 liters of acetone and 210 ml. of water for 15 minutes. It is then allowed to stand for 1 hour, and the supernatant liquid is siphoned off. The precipitate is stirred with 333 ml. of water and 875 ml. of acetone for 15 minutes. After one hour of standing, the supernatant liquid is again removed. Then 790 ml. of acetone and 334 ml. of water are added to the precipitate, and the mixture is stirred for 15 minutes. After one hour of standing, the supernatant is siphoned off.

One hundred fifty ml. of HPS syrup is added slowly to 4 liters of acetone and dispersed with a high-shear mixer. The precipitate of HPS is collected by filtration and air dried for 2 days. The product (48 gm) has an IV of 0.15 and a DS of 0.35.

This HPS product can be used as a cryoprotective agent in the same manner as the HES product of Example I and II.

Example IV

The products of Examples I, II, and III are diluted with water to give a 40% w/v solution of HES or HPS (concentration is determined by optical rotation $[\alpha]_D=178°$). The sodium chloride content is determined by titration with silver nitrate, and sufficient sodium chloride is added to give a final concentration of 0.9% w/v.

The solution is filtered through an 0.8 micron filter with a prefilter on top. The filtrate is collected in containers, which are evacuated, sealed, and sterilized for 30 minutes at 240° C. in an autoclave. The cryoprotectants are then ready for commercial distribution, storage, and use.

Example V

Whole blood, containing a suitable anticoagulant such as ACD, is mixed with a solution of 0.9% sodium chloride containing a quantity (viz. 14—16% w/v) of HES or HPS as prepared in the foregoing Examples, which is sufficient to give *in vitro* recovery of red cells of 90% or greater upon freezing and thawing the blood-HES mixture. This mixture, in a metal container, is placed in a refrigerant bath at a temperature not exceeding about −100° C. and turbulently agitated so that the rate of heat transfer is at least 14,000 B.t.u. per hour per square foot of container surface. (For example, the mixture can be placed in an aluminum container and immersed in liquid nitrogen while shaking at a rate of 200 cpm.) The frozen blood is stored at a temperature of about −100° C. or less. Thawing is accomplished by placing in a 45° C. water bath and agitating at a rate of about 150 cpm.

The *in vitro* recovery of red cells is the percent of nonhemolyzed erythrocytes, determined by 1) measuring the amount of hemoglobin in the supernatant solution obtained after centrifugation of a frozen and thawed blood-HES mixture, 2) measuring the total amount of hemoglobin in the blood-HES mixture before freezing, 3) subtracting the value of step (1) from that of step (2), and (4) expressing the value of

step (3) as a percent of the total hemoglobin.

Preferably, the volume of HES or HPS solution added to the blood before freezing is kept small compared to the volume of blood, to prevent excessive dilution of the blood.

EXAMPLE VI

Whole blood, containing a suitable anticoagulant such as ACD, is centrifuged to separate the red cells from the plasma. The plasma is removed and replaced with a solution of 0.9% sodium chloride containing a quantity of HES or HPS, which is sufficient to give *in vitro* recovery of red cells of 90% or greater upon freezing and thawing, as described in Example IV.

In controlling the inherent viscosity and degree of substitution, various assay procedures can be used. For example, the average molecular weight of the hydroxyethyl or hydroxypropyl starch can be controlled by measuring the inherent viscosity (IV) of the starch during hydrolysis to a definite end point. The inherent viscosity is defined by the relationship:

$$IV = \frac{\ln \frac{t\ solution}{t\ solvent}}{\text{Concentration (gm/100 ml.)}}$$

where t *solution* and t *solvent* are the flow times of the solution and solvent respec- measured in an Ubellohde viscosimeter at solution is 0.8 ± 0.1% starch in IN NaOH, which is the solvent. Flow times are measured in an Ubellohde viscometer at 25.0 ± 0.2° C. The exact starch concentration is determined by measurement of the optical rotation of the solution as follows:

$$\%\ \text{starch (g/100 ml.)} = 0.R. \times 0.61$$

where *O.R.* is the optical rotation in degrees at 20 to 25° C. using the sodium D line, and a 10 cm polarimeter tube.

The DS can be determined by reaction with hydriodic acid. The hydroxyalkyl groups are quantitatively converted to ethylene plus ethyl iodide (or propylene plus isopropyl iodide), which in turn are measured by reaction with bromine and silver nitrate respectively. The procedure used is described by Paul W. Morgan, Industrial and Analytical Chemistry, Analytical Edition *18*, 500—504 (1946).

EXAMPLE VII

Hydroxyethyl starch (HES) and other polysaccharides (for example, dextran) are known to adversely affect blood coagulation when present at a high concentration [see for example, A. A. Garzon *et al*, SURGERY, 62:670 (1967)]. Since one advantage of the present invention is that the preserved frozen blood can be infused without washing out the HES (or HPS) it is highly desirable that the HES used for cryoprotection should be rapidly eliminated from the body, so as to minimize or eliminate the period of high concentration of HES in the blood. It has been found that this can be done by increasing the rate of *in vivo* hydrolysis of the HES, by lowering the DS. It can also be done by lowering the average molecular weight of the HES, by lowering the IV. The limits of DS and IV for effective cryopreservation were therefore determined, as described below.

HES, prepared as described above, was added to ACD-human blood to give final HES concentrations of 12, 15, and 20% w/v. These concentrations were achieved by mixing the following amounts of: (A) 40% HES in 0.9% sodium chloride, and (B) ACD-blood, so as to give 55 ml. of mixture.

Final HES concentration	ml (A)	ml (B)
12%	16.5	38.5
15%	20.6	34.4
20%	27.5	27.5

After standing for 20 minutes at room temperature, the mixture of HES-ACD-blood was placed in a Linde 110 ml. aluminum blood container [described in G. F. Doebbler *et al*, TRANSFUSION *6*: 104 (1966)]. The container was coated with PVP by dipping in a PVP-methanol solution (see the Doebbler reference cited above), and frozen by shaking in liquid nitrogen at 200 cycles per minute for 2 minutes. It was then thawed by shaking at 150 cycles per minute in a 45°C. water bath for 1.5 minutes.

The thawed blood was assayed for erythrocyte recovery (ER) and supernatant hemoglobin (SH) as described in Example V above. Hemoglobin (Hb) was determined by the cyanmethemoglobin method. Saline stability (SS) was determined by diluting the blood in 100 volumes of 0.9% sodium chloride, centrifuging, measuring the supernatant Hb, and expressing it as a percentage of the total Hb in the blood sample.

ER, SH, and SS are indicators of the quality of frozen blood. Well-preserved blood has a low SH, and a high ER and SS.

The effect of DS on cryoprotective action of HES is shown in Table I.

TABLE 1

EFFECT OF DS
IV is 0.15, concentrations 12%, 15%, 29%
(15% values in paranentheses, 20% values in brackets)

DS	SH (mg.%)	ER (%)	SS (%)
0.75	302 (244) [314]	97.2 (97.1) [95.5]	87.3 (85.1) [74.9]
0.61	492 (232)	95.7 (97.4)	72.6 (90.8)
0.36	364 (371) [1446]	97.1 (96.3) [77.6]	89.0 (89.5) [82.9]
0.30	472 (366)	96.0 (96.3)	69.8 (89.7)
0.00	(1500)	(83.2)	(61.1)

It was found that HES of DS in the range 0.30 to 0.75 had a good croyprotective effect provided the concentration was at the proper level in the 12 to 15% range. Pure starch ("HES of DS 0.00") had a poor cryoprotective effect.

The effect of IV on the cryoprotective effect of HES is shown in Table II.

TABLE II

Effect of IV
DS is 0.75, concentrations 12%, 15%
(15% values in parentheses)

IV	SH (mg%)	ER (%)	SS (%)
0.15	302 (244)	97.2 (97.1)	87.3 (85.1)
0.114	412 (292)	96.5 (96.9)	70.5 (84.6)
0.08	494 (378)	95.2 (96.0)	33.7 (35.2)
0.049	1240 (712)	88.4 (91.8)	28.2 (25.0)

It was found that an IV of 0.114 or higher gave good cryoprotective action, but an IV of 0.08 or less gave poor results.

These experiments demonstrate that an IV of about 0.11 and a DS of about 0.30 are the lower limits of effective cryoprotective action of HES for blood. The optimum HES concentration varies between 12 and 15% depending on the IV and DS, as shown in Table I.

WHAT WE CLAIM IS:—

1. A process for the freeze preservation of erythrocytes for subsequent thawing and administration to humans wherein viable erythrocyates are frozen in an aqueous medium, the process comprising incorporating an erythrocyte protective concentration of hydrolyzed starch in said aqueous medium prior to the freezing of said erythrocytes, said starch being a waxy starch composed of at least 90% amylopectin having an ether group degree of substitution of less than 0.50 and above 0.20 per glucose unit, said ether groups being selected from hydroxyethyl and hydroxypropyl groups, and said hydrolysed starch product having an inherent viscosity of 0.11 to 0.27 dl/gm at 25°C.

2. The process of Claim 1 wherein said starch has a degree of substitution of 0.25 to 0.45 ether groups per glucose mole.

3. The process of Claim 1 wherein said aqueous medium is at least in part composed of human blood plasma.

4. The process of Claim 1 wherein said erythrocytes and said aqueous medium are components of whole human blood.

5. The process of Claim 1 wherein said starch has an inherent viscosity of 0.13 to 0.17 dl/gm at 25° C.

6. Frozen whole human blood containing from 13 to 17% w/v of hydrolysed etherified starch, said starch being a waxy starch composed of at least 90% amylopectin having an inherent viscosity of 0.11 to 0.27 dl/gm at 25° C. and having an ether group degree of substitution of less than 0.50 and above 0.20 per glucose unit, said ether groups being selected from hydroxyethyl and hydroxypropyl groups, said blood on thawing being administerable to humans without removal of said starch.

7. The whole blood of Claim 6 wherein said starch has a degree of substitution of 0.25 to 0.45 ether groups per glucose mole.

8. The whole blood of Claim 6 wherein said starch is present in an amount of 14 to 16% w/v.

9. A process for the freeze preservation

of human blood substantially as hereinbefore described with reference to any one of the examples given.

10. A human blood preparation substantially as hereinbefore described with reference to any one of the examples given.

11. A process according to any claim or Claims 1 to 5 and 9 except that said ether groups are hydroxy ethyl groups.

12. The whole blood of any claim of Claims 6 to 8 and 10 except that the ether groups of said waxy starch are hydroxy ethyl groups.

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